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ULTRASTRUCTURE OF EARLY EMBRYONIC SHELL FORMATION IN THE OPISTHOBRANCH GASTROPOD *AEOLIDIA PAPILLOSA*[a]

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Abstract

Early shell formation was examined in embryos of the opisthobranch gastropod *Aeolidia papillosa*. Secretion of the first organic shell material occurs prior to closure of the shell gland lumen, contrary to reports for other molluscan embryos. This difference suggests that in externally shelled gastropods and bivalves initiation of shell secretion may be coincident with narrowing of the shell gland pore rather than with closure of the lumen. The shell growth region was examined ultrastructurally. As no shell material is seen in the shell gland lumen, the shell gland seems not to be actively involved in shell secretion. Initial shell material is secreted only by cells surrounding the shell gland pore. Shell material seems to be added, not in a gap between cells as previously described, but over the apical surface of cells at the growing edge. The growing edge of the shell and the growing edge cells are covered by cytoplasmic extensions arising from the neighboring cells distal to the shell gland. No infoldings of the growing edge cell membranes are seen. The first organic shell material is 20 nm thick, consists of two electron dense layers separated by an electron lucent layer, and is secreted at least 33 hours (5°C) before shell mineralization, as detected by polarizing microscopy.

Introduction

Most molluscs secrete external calcareous shells. Although this secretion begins during early embryogenesis, most of our knowledge of shell formation is derived from studies of post-embryonic molluscs (*e.g.,* Wilbur and Jodrey, 1952; Bevelander and Nakahara, 1969; Wilbur, 1972; Saleuddin, 1974; Weiner and Hood, 1975; Young *et al.,* 1977a, b; Wheeler *et al.,* 1981.). Kniprath (1981) summarized the literature on the development, morphology, and function of the embryonic shell gland and shell field in molluscs; little is known about how, when, and where embryonic shell material is secreted. To date, the only molluscs in which embryonic shell formation has been studied ultrastructurally are the marine bivalves *Mytilus galloprovincialis* and *M. edulis* (Humphreys, 1969; Kniprath, 1980b), the freshwater pulmonate *Lymnaea stagnalis* (Kniprath, 1977), the terrestrial pulmonate *Helix aspersa* (Kniprath, 1980a), the freshwater prosobranch *Marisa cornuarietis* (Kniprath, 1979), and the chitons *Lepidochitona cinera* and *Ischnochiton rissoa* (Haas *et al.,* 1979; Kniprath, 1980c).

The region of ectodermal cells responsible for embryonic shell secretion is called the shell field. Preceding embryonic shell formation in all gastropod and bivalve molluscs, a region of the dorsal shell field invaginates to form the "shell gland"

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(Pelseneer, 1906; chitons lack a true shell gland, see Kniprath, 1981). It is because this invagination always forms in externally shelled species, that it has been assumed to have an active function in shell formation. The invaginated region has been referred to as the shell gland since 1873 (see Kniprath, 1979) although its actual role in shell formation has been little studied and remains unclear. After the shell gland invaginates, its lumen "closes" (narrows to a canal open to the outside through a pore; see Kniprath, 1979, Figs. 2a, b; 1980, Fig. 1e). The shell gland later evaginates or spreads back to a non-invaginated shell field.

In 1979 Kniprath outlined three aspects of early shell formation that were in a state of confusion and that warranted further examination: 1.) At what developmental stage of the shell gland is the first shell material secreted? 2.) Which cells secrete the first shell material? and 3.) How does evagination of the shell gland proceed. Although several authors have addressed the first two problems (*e.g.*, Humphreys, 1969; Kniprath, 1980a, b), conflicting results have been presented and a clear description of the cells at the leading or growing edge of the embryonic shell is lacking. Also absent are precise date on the shape of the shell gland at the time of secretion of the first organic shell material.

The present work provides the first ultrastructural description of embryonic shell formation in a marine gastropod. In this paper I describe when and where the first organic shell material is observed in embryos of the nudibranch *Aeolidia papillosa*. The fine structure of the first shell material and of the cells at the early growing edge of the shell are also examined.

Materials and Methods

Reproductively active specimens of *Aeolidia papillosa* were collected subtidally near Nahant, MA using SCUBA and were placed in a flow-through sea water aquarium. Adults and young were thus exposed to natural temperature (5°C) and salinity (30‰) conditions. Egg masses laid on the aquarium walls soon after incarceration of the adults were allowed to harden for a few days before they were carefully removed and placed in wide-mesh baskets suspended in the aquarium.

Capsules containing embryos were removed mechanically from the egg masses and examined under a compound microscope to confirm synchrony, normality, and stage of embryonic development. Polarizing microscopy (pieces of polarizing film set at maximum extinction) was used to ascertain initiation of shell formation since the initial shell material is not detectable with standard light microscopy. Birefringence in the shell field observed with polarizing microscopy indicates shell mineralization rather than presence of the organic portion of the shell since treatment of embryos with the calcium chelator EGTA (10 m*M* ethylene-glycol-bis-N,N′-tetra acetic acid) resulted in loss of the birefringence. Since secretion of organic shell material precedes shell mineralization, by the time birefringence was detectable I knew that the first organic shell material had already been secreted. Because of the functional relationship between the organic materials and inorganic mineralization, timing of the various developmental stages is given in hours preceding detectable birefringence (Fig. 19).

Once gastrulation had begun and until calcareous shell material was detectable with polarizing microscopy, embryos within their egg capsules were removed periodically from the egg masses and fixed. Embryos were held in fixative in a refrigerator up to 3 days, until the last sample was fixed. A variety of primary fixatives were tried; the best results were obtained with 3% glutaraldehyde, 1% formaldehyde with paraformaldehyde (Karnovsky, 1965), 3% NaCl, 4.5% sucrose in 0.1 *M* phos-

phate buffer, with dimethylsulfoxide added to aid penetration of the fixative (pH 7.4). Embryos were washed at room temperature in 0.1 *M* phosphate buffer with 8% sucrose and post-fixed in 2% OsO_4 in 0.2 *M* phosphate buffer for 1 h. The tissue was dehydrated in a graded series of ethanol to 100% and was infiltrated with and embedded in Spurr low viscosity embedding medium (Spurr, 1969).

Embryos were sectioned at random orientation since they could not be oriented. For light microscopy, from 10–72 embryos at each stage were serially sectioned (0.5–1.0 μm). Sections were cut using glass knives, mounted onto glass slides, and stained with Richardson's stain (Richardson *et al.*, 1960). For transmission electron microscopy (TEM), thin sections were cut on a Sorvall MT-2B ultramicrotome using glass or diamond knives, mounted onto copper grids, and stained 15 minutes in saturated aqueous uranyl acetate followed by lead citrate. For TEM localization of periodate-reactive carbohydrates, thin sections were mounted onto gold grids and exposed sequentially to periodic acid (PA), thiosemicarbazide (TSC), and silver proteinate (SP); appropriate controls were run simultaneously (Thiéry, 1967; Porter and Rivera, 1979). Thin sections were examined and photographed on an RCA EMU-4 transmission electron microscope.

Results

Initiation of shell secretion

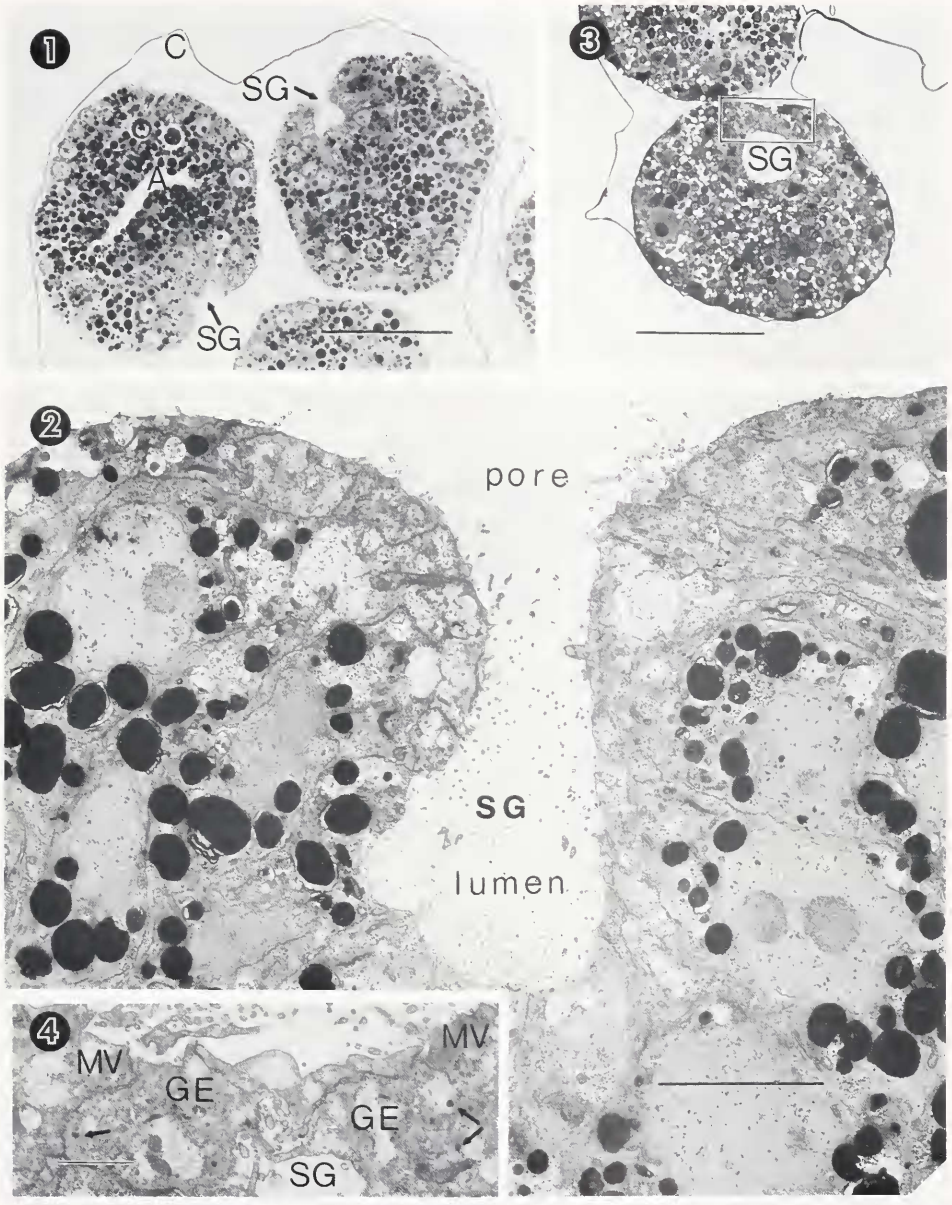
The shell gland invagination is present by 2 days (5°) prior to first detectable birefringence in embryos of *Aeolidia papillosa* (Figs. 1, 2). At this stage embryonic ciliation is just visible with a compound microscope; the embryos move slightly inside their capsules but do not spin actively. Based on the large size of the shell gland pore (about 17 μm) and irregular outline of the shell gland lumen, the shell gland seems to be still forming. No secreted shell material is evident at this stage with transmission electron microscopy although the dense granules believed to be involved in shell formation (see Fig. 10) are already present. At all stages of development, the shell gland lumen is lined with scattered microvilli.

In embryos fixed three hours later, at 43 hours before birefringence is observed, the shell gland lumen is more circular in section and up to 26 μm wide and 30 μm deep (Fig. 3). Also the shell gland pore has become smaller. The smallness of the pore and the fact that the embryos are insufficiently differentiated to be oriented prior to sectioning makes it difficult to obtain sections passing through both the pore and the lumen of the shell gland at this and all later stages. No secreted shell material is observable with transmission electron microscopy in embryos at this stage (Fig. 4).

In embryos fixed 33 hours before the first birefringence, the shell gland has changed to a more oval shape with a shell gland neck that is narrower than the rest of the shell gland lumen (Figs. 5, 6). It is at this stage that the first organic shell material is observed. The shell material covers the opening of the shell gland pore. Additions of new organic material are made at the growing edge, away from the shell gland (Fig. 6).

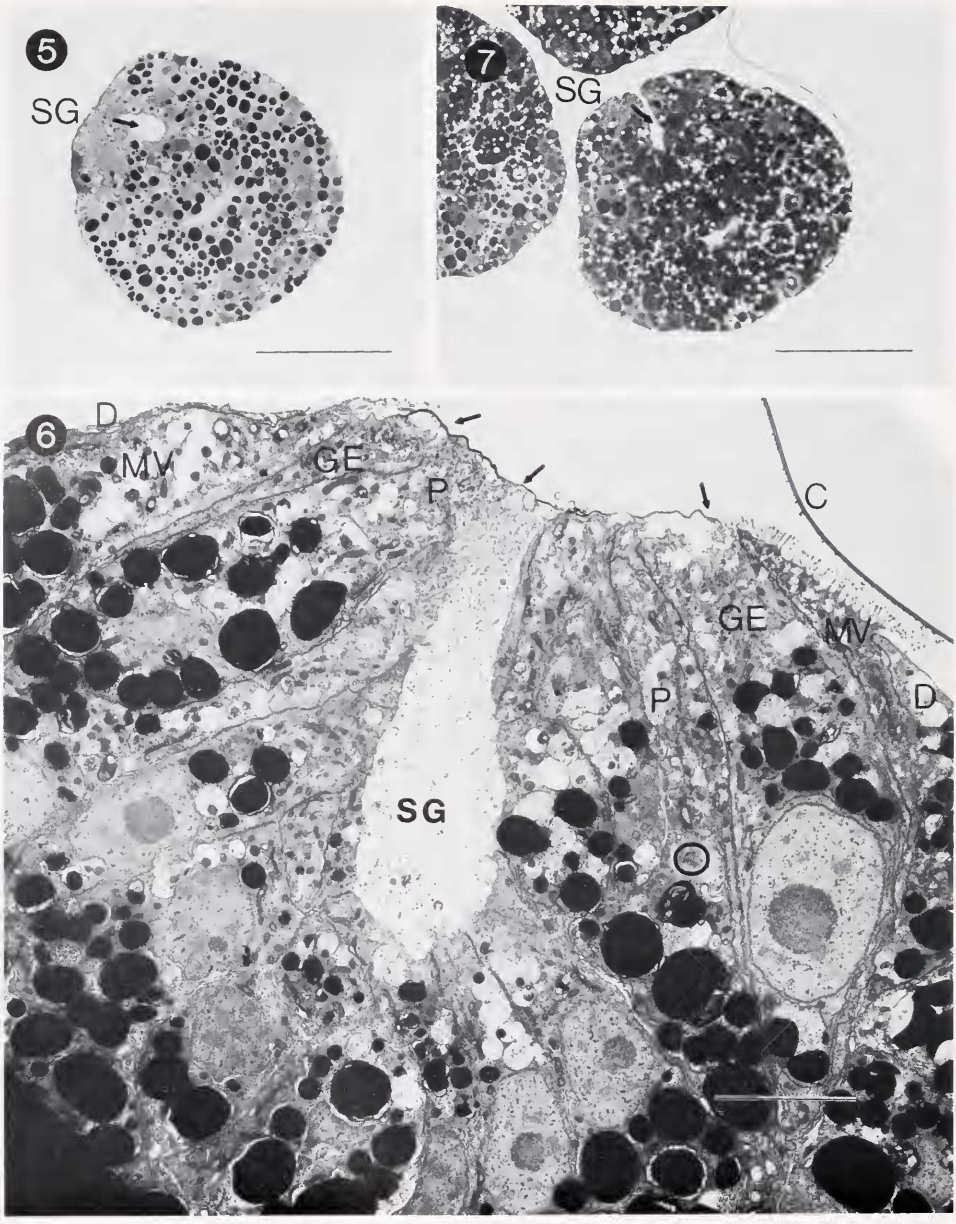
Two areas of shell growth are seen in each section. The cells directly beneath the zone of shell growth are referred to as "GE cells" because of their proximity to the growing edge (GE). The cells adjacent to the GE cells but distal to the shell gland are referred to as "MV cells" because of their characteristic abundance of microvilli (see Figs. 20, 21).

At 23 hours prior to the first detectable shell mineralization, the shell gland has closed to a narrow canal but is still open to the outside through a small pore (Figs.



FIGURES 1, 2. Micrographs of sections through *Aeolidia papillosa* embryos fixed 46 h prior to shell mineralization (5°C). FIGURE 1. Light micrograph. Embryos are within egg capsule (C) and show shell gland (SG) and archenteric (A) invaginations. Bar = 50 μm. FIGURE 2. Transmission electron micrograph (TEM) showing pore and lumen of shell gland (SG) prior to shell secretion. Bar = 5 μm.

FIGURES 3, 4. Micrographs of sections through embryos 43 h prior to shell mineralization. The shell gland (SG) is open to outside through a pore, not visible here or in Figure 5 due to sectioning angle. FIGURE 3. Light micrograph. Bar = 50 μm. FIGURE 4. TEM of region similar to box in Figure 3, showing growing edge cells (GE) with electron dense granules (arrows), and microvilli-bearing cells (MV). Bar = 1 μm.



FIGURES 5, 6. Micrographs of sections through embryos fixed 33 h prior to shell mineralization. FIGURE 5. Light micrograph. Bar = 50 μm. FIGURE 6. TEM of newly secreted shell material (arrows) lying over the pore of the shell gland (SG), the proximal cells (P), and growing edge cells (GE). Also shown are microvilli-bearing cells (MV), distal cells (D), and one cluster of vesicles present in proximal cells (circle). Bar = 5 μm.

FIGURE 7. Light micrograph of section through embryos fixed 23 h prior to shell mineralization. The shell gland (SG) has "closed" to a narrow canal. Bar = 50 μm.

7, 19). The lumen of the shell gland canal is still lined with scattered microvilli but the number of microvilli seen in any section is greater after narrowing of the shell gland lumen. This apparent increase in abundance of microvilli may reflect decreased distance between cells lining the lumen rather than an actual increase in number of microvilli. Although Figures 8 and 11–18 are all from embryos fixed 23 h prior to mineralization, at this stage MV cells, GE cells, and the shell itself have the same morphological characteristics observed in embryos fixed 10 hours earlier. The morphology of these cells and of the secreted shell material are described below.

Morphology of the shell growth region

The zone of shell growth is near the apical surface of the GE cells. The GE cells are columnar, have rough endoplasmic reticulum associated with sub-basal nuclei, and have fields of periodic acid–thiosemicarbazide–silver proteinate (PA-TSC-SP) positive material, presumed to be glycogen. These cells are readily identified by the presence of numerous membrane-bound granules (Figs. 8, 9). In section the granules are either circular or oblong and have a maximum length of 200 nm (Fig. 10). The granules are frequently seen in association with Golgi apparati just apical to the nucleus (Fig. 11); often near the cell apices (Fig. 8); occasionally within apical cytoplasmic extensions (Fig. 9); but never outside of the cell.

Electron cytochemistry is currently being utilized to determine if the granules contain potential organic or inorganic shell components. The major organic shell component in molluscs is protein (Wilbur, 1972), but no stains are specific for protein (Hayat, 1970). Because polysaccharides are also present in molluscan shells (Wilbur, 1972), the PA-TSC-SP procedure was used. Preliminary tests with the PA-TSC-SP procedure indicate that the granules do not contain carbohydrates oxidizable with periodic acid. The granules are electron lucent in glutaraldehyde-osmium fixed sections, but are very electron dense after sequential staining with uranyl acetate and lead citrate. No distinct substructure was observed in stained or unstained granules at a magnification of 500,000×.

At all stages prior to mineralization, the shell consists of two electron dense layers separated by an electron lucent layer or gap (Fig. 12). In embryos fixed 33 hours prior to detectable mineralization, the shell material seen in section was up to 10 μm long (following all contours) and 22 nm thick. Clusters of small vesicles, most about 15–60 nm in diameter, are associated with the outer surface of the shell (Figs. 13, 14). These clusters appear to be randomly scattered.

The growing edge of the shell either abruptly terminates (Figs. 9, 16) or consists of small electron dense particles (Figs. 8, 18, 20). Regardless of its exact morphology, the edge of the newly formed shell material is always located on the apices of the GE cells and never between the lateral plasma membranes of the GE cells and the neighboring MV cells. No secretory infoldings of the lateral plasma membranes of the GE cells were observed.

The proximal cells, those cells adjacent to the GE cells and proximal to the shell gland, occasionally have infolded apical plasma membranes (Fig. 17). These cells also occasionally contain electron dense granules as described in the GE cells. One consistent feature of these cells is their association with apical-lateral intercellular spaces lined with microvilli. A space was consistently observed between the GE cells and proximal cells (Figs. 16–18, 20). The shell extending over this intercellular space is almost entirely separated from it by cytoplasmic extensions arising from the cells lining the space (Figs. 16, 18). These cytoplasmic extensions are in intimate contact with the inner surface of the shell (Figs. 16, 18). Groups of small uncoated vesicles

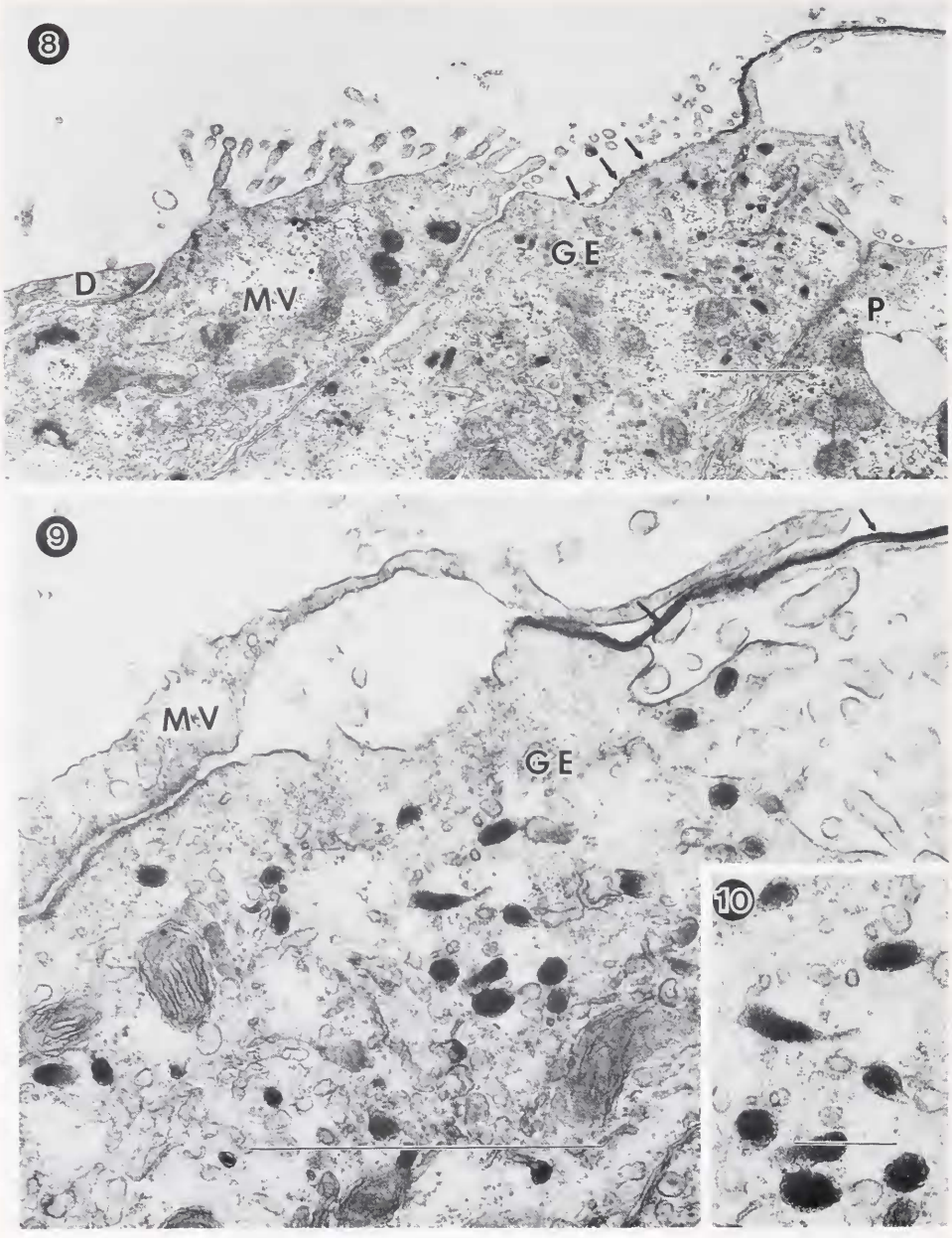
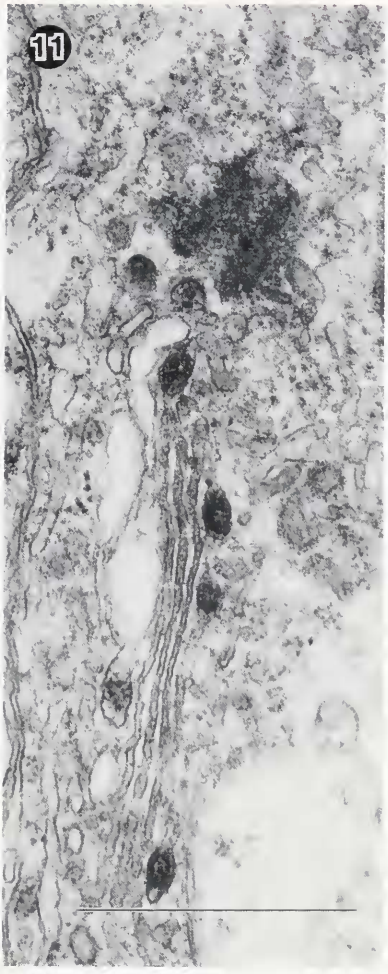
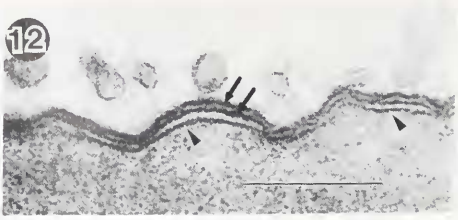


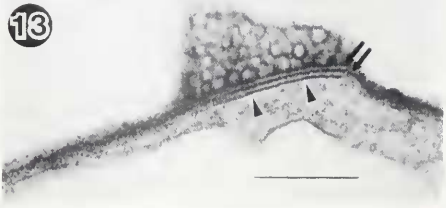
FIGURE 8. Transmission electron micrograph of apices of: cell distal to shell gland (D), microvilli-bearing cell (MV), growing edge cell (GE), and proximal cell (P). The growing edge of the shell lies over the GE cell and here consists of small electron dense particles (arrows). Bar = 1 μm.

FIGURE 9. Apex of growing edge cell (GE), characterized by electron dense granules; from embryo fixed 33 h prior to mineralization. A cytoplasmic extension of the microvilli-bearing cell (MV) lies over the growing edge of the shell (arrows). Bar = 1 μm.

FIGURE 10. Electron dense granules in GE cell, from Figure 9. Bar = 200 nm.







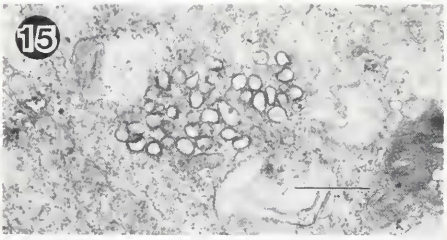


FIGURE 11. Electron dense material associated with Golgi complex. Bar = 500 nm.

FIGURE 12. Section showing two electron dense layers of shell (arrows) lying close to plasma membrane (arrowheads) of shell field cell. Sections through microvilli are shown at top. Bar = 200 nm.

FIGURES 13, 14. Clusters of small vesicles associated with outer surface of shell (arrows). FIGURE 13. The dense layer below the shell is the plasma membrane (arrowheads). Bar = 200 nm. FIGURE 14. Bar = 1 μm.

FIGURE 15. Small vesicles seen in proximal cells. Bar = 500 nm.

circular to pear-shaped in profile and about 100 nm in diameter are present in the proximal cells (Figs. 6, 15). These vesicles have lucent cores but are larger and have a much denser border than the vesicles present on the outer surface of the shell.

Only one MV cell with numerous microvilli is observed in section at each growing edge (Figs. 6, 16). In comparison, the distal cells (cells adjacent to MV cells but distal to shell gland), never have more than a few scattered microvilli (Figs. 6, 8, 17, 21). The MV cells do not contain the electron dense granules typical of the GE cells but both the MV cells and GE cells have numerous mitochondria apically. The MV cells are joined apically to the GE cells by zonulae adhaerens and septate

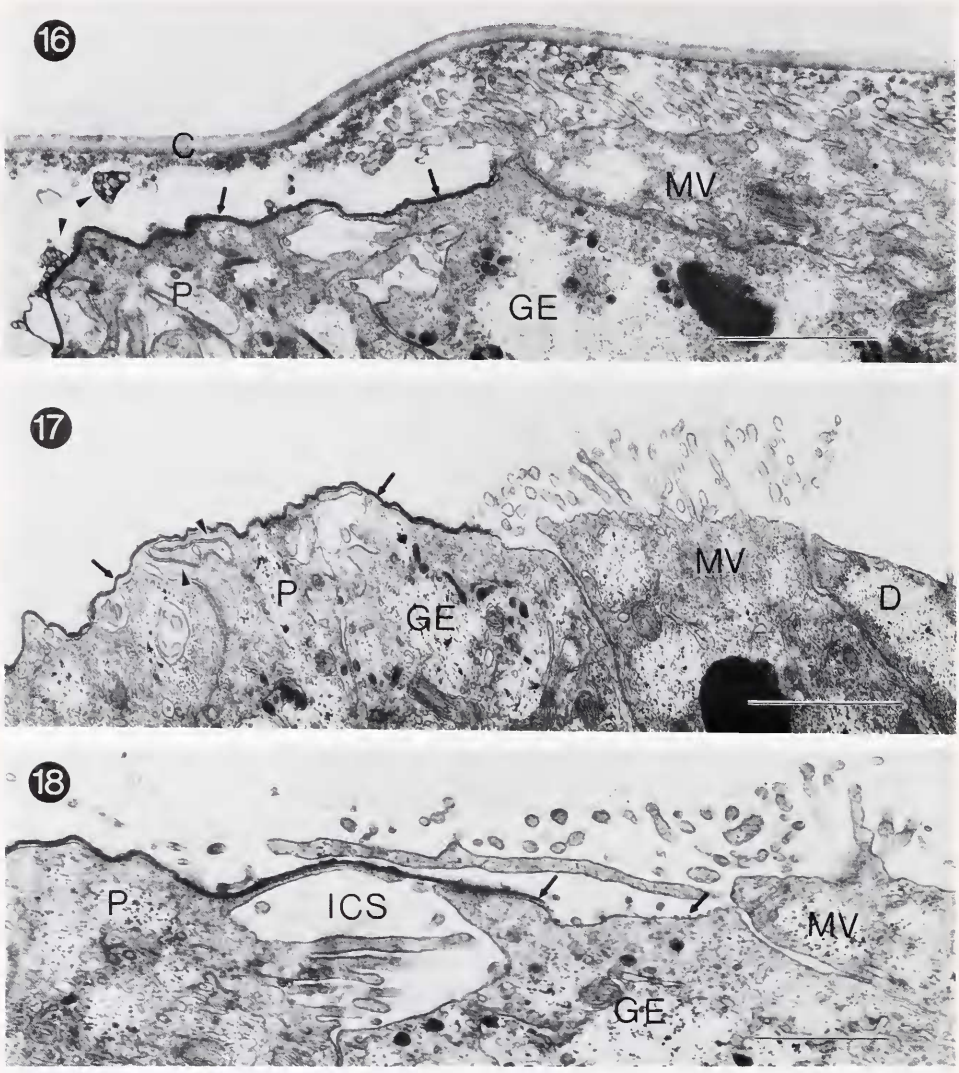


FIGURE 16. The microvilli of the MV cell lean over the growing edge cell (GE) and over the growing edge of the shell. Two clusters of vesicles are shown (arrowheads); rarely were clusters seen not in contact with the outer shell surface (arrows). P = proximal cell; C = capsule. Bar = 1 μm.

FIGURE 17. Infoldings (arrowheads) of the apical plasma membranes of the proximal cells (P) were observed rarely; no infoldings of other shell fields cells were observed. The growing edge of the shell is covered by abundant microvilli of the microvilli-bearing cell (MV). The distal cell (D) has only sparce microvilli. Arrows = shell. Bar = 1 μm.

FIGURE 18. An intercellular space (ICS) occurs between the proximal cells (P) and growing edge cells (GE). Some sections show long cytoplasmic extensions from the microvilli-bearing cells (MV) covering the growing edge of the shell (arrows). Bar = 1 μm.

desmosomes. The microvilli of the MV cells tend to lean over the GE cells and the growing edge of the shell (Figs. 16, 17). Long cytoplasmic extensions that arise from the inner edges of the MV cells also reach over the growing edge of the shell and may completely cover the apical surfaces of the GE cells (Figs. 9, 18).

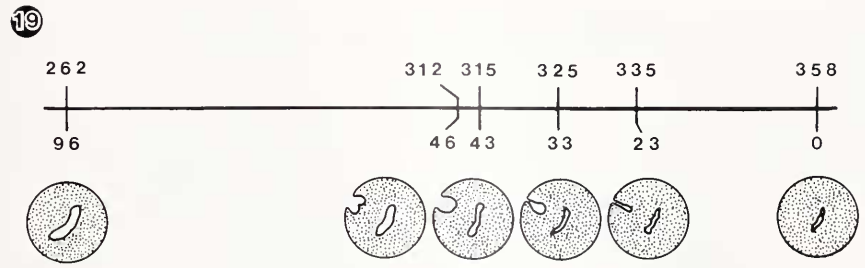


FIGURE 19. Schematic diagram of changes in shell gland morphology related to time (hours) after oviposition (top scale) and time prior to detection of shell mineralization (bottom scale), 5°C. Drawings represent sections through embryos at gastrulation and as in Figures 1, 3, 5, and 7. Not to scale.

DISCUSSION

Timing of first shell secretion

The first shell material in embryonic molluscs is secreted sometime during the existence of the shell gland (Cather, 1967; Demian and Yousif, 1973; Kniprath,

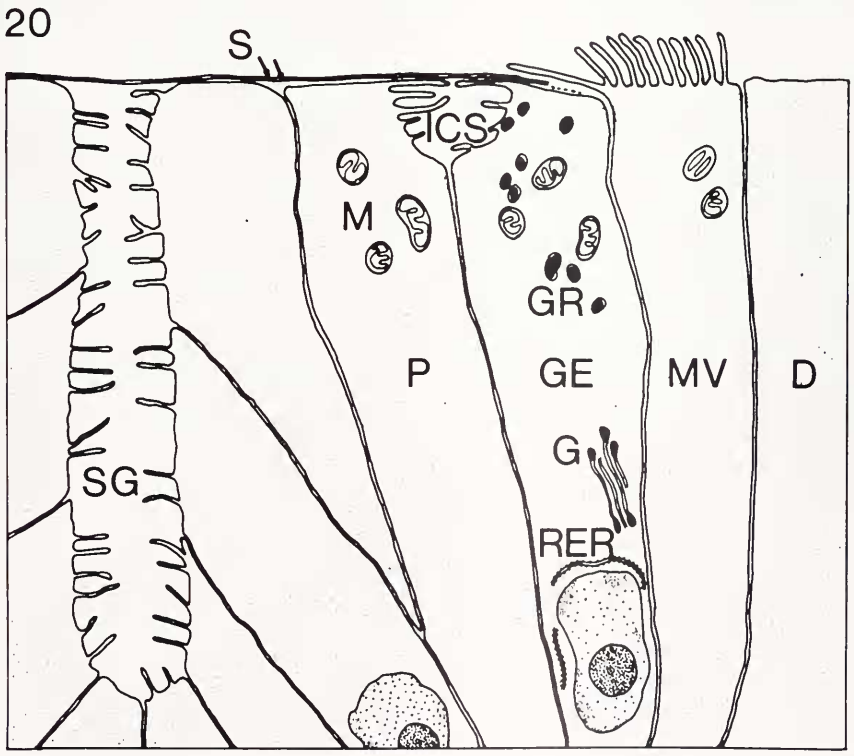


FIGURE 20. Schematic diagram showing arrangement of shell and early shell field cells, at about 30 h prior to detection of shell mineralization. The shell (S) consists of two electron dense layers (arrows). At its growing edge, the shell consists of small electron dense particles lying on the apical surface of the growing edge cell (GE) and is covered by cytoplasmic extensions arising from the microvilli-bearing cell (MV). Also shown are proximal (P) and distal (D) shell field cells, named in terms of their proximity to the shell gland (SG). M = mitochondria. GR = granules. G = Golgi complex. RER = rough endoplasmic reticulum. ICS = intercellular space. Not to scale.

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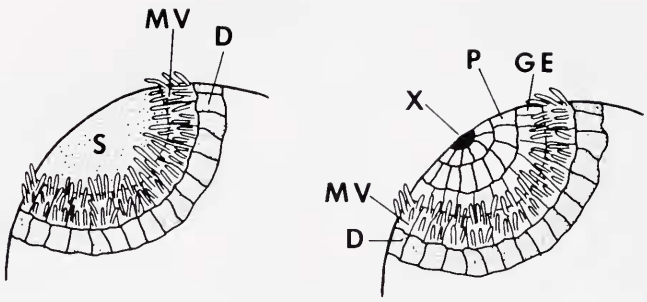

FIGURE 21. Schematic diagram of hypothetical arrangement of shell and early shell field cells, in surface view. At the left the shell (S) covers the underlying cells and extends to the microvilli-bearing cells (MV). The same region is redrawn (right) with the secreted shell material removed to reveal underlying cells and pore of the shell gland (X). P = proximal cells. GE = growing edge cells. D = distal cells. Not to scale.

1977). However, the shell gland of developing embryos is present for hours and goes through several morphologically distinct stages or shape changes. Only Kniprath (1977, 1979, 1980b) has specifically examined the morphological stage of the shell gland at which shell secretion begins.

In *Aeolidia papillosa* the first organic shell material is secreted at least 10 hours before the shell gland "closure" stage (see Fig. 19) and at least 33 hours prior to detection of shell mineralization. The electron dense granules believed to contain components of organic shell material were present at least 13 hours prior to observation of first secreted shell material and 23 hours prior to shell gland closure. This is in contrast to the findings of Kniprath (1981) who stated that in the species he examined the cells of the shell field "do not synthesize anything for secretion before the closure stage". It is uncertain whether the cells lining the shell gland lumen ever become tightly apposed in *Aeolidia papillosa.*

In contrast, in the snails *Lymnaea stagnalis* (Kniprath, 1977) and *Marisa cornuarietis* (Kniprath, 1979), the shell gland lumen closes to a canal prior to shell secretion. In the mussel *Mytilus galloprovincialis* (Kniprath, 1980b) the walls of the shell gland seem to close so tightly that not even a narrow canal is detectable with transmission electron microscopy. In that species, the shell gland lumen is apparently completely gone prior to shell secretion. Thus closure of the shell gland lumen precedes shell secretion in all three of these species, but not in *Aeolidia papillosa.*

Secretion of the first shell material while the shell gland is still open in *Aeolidia papillosa* demonstrates that closure of the shell gland lumen is not requisite to initiation of shell secretion. Instead, the size of the pore of the shell gland may be the important factor, especially if the shell material is first secreted over the pore rather than along the lining of the shell gland lumen. In all of the above species the shell gland pore becomes smaller prior to shell secretion. Presumably a small pore would be easier to seal over with shell material than would a large pore.

Identification of shell secreting cells

The embryonic shell of molluscs is often said to be secreted by the "shell gland" (Fretter and Graham, 1962; Raven, 1966; Jablonski and Lutz, 1980), a term which has different meanings to different authors. In many cases, general statements about the "shell gland" are in fact references to the entire shell-secreting epithelium, (*i.e.,*

the shell field) regardless of its morphology. Originally the term was applied by Ray Lankester just to the invagination (Pelseneer, 1906), not to the entire shell field. The term has more recently also been defined as the calcifying invagination of the ectoderm (Waller, 1978). As used in the present work the term shell gland strictly refers to the invaginated region of the shell field without reference to function. Thus the shell gland is the center of the early shell field. After shell gland evagination or spreading, the cells that once lined the shell gland lumen are still shell field cells (Kniprath, 1979, 1981).

The present ultrastructural evidence demonstrates that in *Aeolidia papillosa* the first organic portion of the shell is secreted only by the non-invaginated shell field cells around the shell gland pore. No substances resembling shell material were ever observed within the lumen of the shell gland. Thus, it is clear that the shell gland *sensu stricto* does not secrete the embryonic shell in *Aeolidia papillosa*.

These results support the electron microscopic work of Kniprath (1977, 1979, 1980a, 1980b) on *Lymnaea stagnalis, Marisa cornuarietis, Helix aspersa,* and *Mytilus galloprovincialis*. Kniprath reported that the first shell material is secreted solely by a ring of cells surrounding the shell gland pore while the invaginated cells of the shell gland remain nonsecretory until calcium secretion begins. Possibly the invagination of these cells while they are nonsecretory serves to prevent a large hole from forming in the center of the shell, an idea suggested previously by several workers (see Haas *et al.,* 1979; Kniprath, 1979).

It should be noted that earlier work based on light microscopy (*e.g.,* Cather, 1967, on *Ilyanassa obsoleta;* Demian and Yousif, 1973, on *Marisa cornuarietis;* Raven, 1975, on *Lymnaea stagnalis*) produced results conflicting with later studies utilizing TEM (*e.g,* Kniprath, 1977, on *Lymnaea stagnalis;* Kniprath, 1979, on *Marisa cornuarietis;* present study on *Aeolidia papillosa*). These conflicting results probably do not reflect biological differences. With light microscopy the shell material was observed extending into the shell gland lumen and therefore was believed to have been secreted there. With transmission electron microscopy the first shell material is seen only outside the shell gland (lying over the shell gland pore). These differences may reflect several factors. First, earlier authors may have been unable to detect the very first shell material with light microscopy. The initial shell material, because of its thinness, may not be detectable with light microscopy until it separates from the underlying epithelium. After separation the shell material might then fold down into the lumen, giving the impression that it was secreted there. Second is the possibility that the earliest shell material might be dislodged from excapsulated or non-encapsulated embryos during handling for fixation and dehydration. If the very early shell material can be dislodged by handling then the shell would not be detected when it is initially secreted. In the present study all embryos of *Aeolidia papillosa* were fixed and dehydrated within their capsules. A third factor that might have lead to these different conclusions concerning the timing and location of initial shell secretion is sampling (fixation) frequency. In some species studied the frequency of sampling may have been low relative to rate of shell development, so that the earliest stages of shell formation may have been missed. Sampling more frequently relative to developmental rate should help resolve some or all of these issues.

It is not clear whether the various cell types seen in the early shell field maintain their respective functions throughout evagination or spreading of the shell gland. In *Aeolidia papillosa* the cells at the growing edge are seen further and further from the shell gland lumen as evagination proceeds, suggesting that these cells are merely migrating. Whether or not the cells change function following evagination as the shell field grows into a distinct mantle is yet to be documented.

Certainly, further studies of other molluscan species are required to determine the range of shell gland morphologies and to elucidate the role of the shell gland cells *versus* that of other cells of the shell field. If such studies demonstrate that the invaginated cells have no role in secretion of either organic or inorganic shell components, what is now called the shell gland might be better referred to as the shell field invagination.

Site of early shell secretion

It is well known that regions where biomineralization proceeds are sealed off from chemical influences of the surrounding environment (Wilbur, 1972; Clark, 1976). Clark (1976) reviewed three main approaches to marginal calcification in post-larval invertebrates, two of which deserve further mention here. First, in some invertebrates such as scleractinian corals, a marginal fold of tissue drapes over the growing margin, isolating it from sea water. Secondly, in many molluscs and brachiopods, periostracum is secreted in a marginal fold and isolates the underlying region of shell mineralization. Also, the shell material in molluscs may be securely anchored to the apices of the secreting cells (Chétail and Krampitz, 1982), thus isolating the inner surface of the shell from the external medium. In molluscan embryos the location of the growing edge and method(s) of sealing it off have not been established.

Few authors have examined the location of the early growing edge in molluscan embryos on an ultrastructural level. Humphreys (1969) briefly described embryonic shell formation in the mussel *Mytilus edulis,* stating that the growing edge of the shell was intracellular. He suggested that it undercut the cilia and microvilli of the cell apices, all of which were subsequently sloughed off. However, Kniprath (1980b) has determined that the first and outermost shell material of *Mytilus galloprovincialis* is laid down extracellularly and seems to be protected from the surrounding medium by a thick glycocalyx and by microvilli of the adjacent cells. He also reported that the growing edge in *M. galloprovincialis* lies in an intercellular gap, sometimes down to the desmosome, and that infoldings of the lateral plasma membranes in this region seem to secrete materials that thicken the shell pellicle. This intercellular gap may also serve to seal off the growing edge.

Ultrastructural observations on *Aeolidia papillosa* confirm that the shell is laid down extracellularly. However, instead of forming in a lateral intercellular gap, the embryonic shell of *A. papillosa* seems to be produced on the apical surface of the GE cells. No infolding of the lateral plasma membranes was seen in this area, and no shell material in addition to the two dense lamellae was observed in regions of the shell distant from the growing edge. Thus additions to the shell in this species seem to occur solely over the cell apices, where the growing edge is potentially exposed to the surrounding environment.

Two factors may be involved in sealing off the shell edge of *Aeolidia papillosa.* First, the shell in this region is closely applied to the underlying cells and seems to be secured to the cell apices (Figs. 16, 18), while in regions away from the growing edge the shell is often separated from the underlying cells by a gap. Secondly, the MV cells may have a role in sealing off the growing edge. The microvilli of these cells are angled towards the GE cells (Fig. 16), and long cytoplasmic extensions arising from the proximal edges of the MV cells lie over the growing edge of the shell (Figs. 9, 18). Haas (1976), Haas *et al.* (1979), and Kniprath (1980c) have observed a similar situation and reached a similar conclusion for shell plate formation in chitons. Haas (1976) suggests that the microvilli may form "a barrier which

controls the growth of the tegmental crystals". Kniprath (1980c) provided support for this idea by his observation that the first trace of mineral detectable under polarizing microscopy was seen at exactly the stage of development where the large flat villi from the distal edges of neighboring cells overlapped and closed off the crystallization space. Although the MV cells may serve other functions (transport?) than isolation of the growing edge of the shell in *Aeolidia papillosa,* it is doubtful that they secrete the organic shell material since they lack the dense granules presumed to contain organic shell components and since the growing edge of the shell lies over a different cell type.

Preliminary studies (Eyster, unpubl.) on the development of several other opisthobranch species show that the growing edge of the shell of these species also lies over the apical surfaces of the GE cells and that it is covered by cytoplasmic extensions arising from the MV cells. Possibly, presence of the growing edge on the potentially exposed cell apices of developing opisthobranch embryos reflects protection from the surrounding medium afforded by the embryonic capsules. Attempts to mechanically remove the capsules surrounding young embryos failed although the same procedures worked on embryos ready to hatch; embryos removed from the egg mass but left within their capsules developed normal shells. Preliminary attempts to examine the early growing edge in *A. papillosa* with scanning electron microscopy have proved unproductive due to the obscuring of the embryonic surface with precipitated components of the fluid held inside the capsule. Further studies are required to determine whether these reported differences in early shell development between opisthobranch gastropods and other taxa have phylogenetic significance.

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LITERATURE CITED

Bevelander, G., and H. Nakahara. 1969. An electron microscope study of the formation of the nacreous layer in the shell of certain bivalve molluscs. *Calcif. Tiss. Res.* **3**: 84–92.

Cather, J. N. 1967. Cellular interactions in the development of the shell gland of the gastropod, *Ilyanassa. J. Exp. Zool.* **166**: 205–224.

Chétail, M., and G. Krampitz. 1982. Calcium and skeletal structures in molluscs: concluding remarks. Proc. Seventh International Malacological Congress. *Malacologia* **22**(1–2): 337–339.

Clark, G. R., II. 1976. Shell growth in the marine environment: Approaches to the problem of marginal calcification. *Am. Zool.* **16**: 617–626.

Demian, E. S., and F. Yousif. 1973. Embryonic development and organogenesis in the snail *Marisa cornuarietis.* (Mesogastropoda: Ampullariidae). IV. Development of the shell gland, mantle and respiratory organs. *Malacologia* **12**(2): 195–211.

Fretter, V., and A. Graham. 1962. *British Prosobranch Molluscs. Their Functional Anatomy and Ecology.* Bartholomew Press, Dorking, Great Britain. 755 pp.

Haas, W. 1976. Observations on the shell and mantle of the Placophora. Pp. 389–402 in *The Mechanisms of Mineralization in the Invertebrates and Plants,* N. Watabe, and K. M. Wilbur, eds. Belle W. Baruch Library in Marine Science, 5. Univ. South Carolina Press, Columbia, 461 pp.

Haas, W., K. Kriesten, and N. Watabe. 1979. Notes on the shell formation in the larvae of the Placophora (Mollusca). *Biomineralization* **10**: 1–8.

Hayat, M. A. 1970. *Principles and Techniques of Electron Microscopy. Biological Applications, I.* Van Nostrand Reinhold, Co., New York. 412 pp.

HUMPHREYS, W. J. 1969. Initiation of shell formation in the bivalve, *Mytilus edulis*. *Proc. Electr. Microsc. Soc. Am.*, Twenty-seventh Ann. Meeting: 272–273.

JABLONSKI, D., AND R. A. LUTZ. 1980. Molluscan larval shell morphology. Ecological and paleontological applications. Pp. 323–377 in *Skeletal Growth of Aquatic Organisms*, D. C. Rhoads and R. A. Lutz, eds. Plenum Pub. Co., New York. 750 pp.

KARNOVSKY, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell. Biol.* **27**: 137 A.

KNIPRATH, E. 1977. Zur Ontogenese des Schalenfeldes von *Lymnaea stagnalis*. *Wilhelm Roux's Arch.* **181**: 11–30.

KNIPRATH, E. 1979. The functional morphology of the embryonic shell-gland in the conchiferous molluscs. *Malacologia* **18**: 549–552.

KNIPRATH, E. 1980a. Sur la glande coquilliere de *Helix aspersa* (Gastropoda). *Arch. Zool. Exp. Gén* **121**: 207–212.

KNIPRATH, E. 1980b. Larval development of the shell and the shell gland in *Mytilus* (Bivalvia). *Wilhelm Roux's Arch.* **188**: 201–204.

KNIPRATH, E. 1980c. Ontogenetic plate and plate field development in two chitons, *Middendorffia* and *Ischnochiton*. *Wilhelm Roux's Arch.* **189**: 97–106.

KNIPRATH, E. 1981. Ontogeny of the molluscan shell field: a review. *Zool. Scri.* **10**: 61–79.

PELSENEER, P. 1906. Part V. Mollusca. In: 1906. *A Treatise on Zoology*, Edwin Ray Lankester, ed. Adam and Charles Black, London. 355 pp.

PORTER, K. J., AND E. R. RIVERA. 1979. An ultrastructural cytochemical analysis of mucoid secretory granules in nudibranch epidermis. *Proc. Electron Microsc. Soc. Am.*, 37th Ann. Meeting: 302–303.

RAVEN, C. P. 1966. *Morphogenesis: The Analysis of Molluscan Development*. Pergamon Press, New York. 366 pp.

RAVEN, C. P. 1975. Development. Pp. 367–400 in *Pulmonates I. Functional Anatomy and Physiology*, V. Fretter and J. Peake, eds. Academic Press, New York. 417 pp.

RICHARDSON, K. C., L. JARRETT, AND E. H. FINKE. 1960. Embedding in epoxy resin for ultrathin sectioning in electron microscopy. *Stain Technol.* **35**: 313–323.

SALEUDDIN, A. S. M. 1974. Ultrastructural studies on the structure and formation of the periostracum in *Helisoma* (Mollusca). Pp. 309–337 in *The Mechanisms of Mineralization in the Invertebrates and Plants*, N. Watabe, and K. M. Wilbur, eds. Belle W. Baruch Library in Marine Science, 5. Univ. South Carolina Press, Columbia. 461 pp.

SPURR, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**: 31–43.

THIÉRY, J. P. 1967. Mise en evidence des polysaccharides sur coupes fines en microscopie electronique. *J. Microsc.* **6**: 987–1018.

WALLER, T. R. 1978. Formation of a posterodorsal notch in larval oyster shells and the prodissoconch—I/II boundary in the Bivalvia. *Bull. Am. Malacol. Union, Inc.*, 1978: 55–56.

WEINER, S., AND L. HOOD. 1975. Soluble protein of the organic matrix of mollusk shells: a potential template for shell formation. *Science* **190**: 987–989.

WHEELER, A. P., J. W. GEORGE, AND C. A. EVANS. 1981. Control of calcium carbonate nucleation and crystal growth by soluble matrix of oyster shell. *Science* **212**: 1397–1398.

WILBUR, K. M. 1972. Shell formation in mollusks. Pp. 103–145 in *Chemical Zoology*, 7, M. Florkin, and B. T. Scheer, eds. Academic Press, London. 567 pp.

WILBUR, K. M., AND L. H. JODREY. 1952. Studies on shell formation I. Measurement of the rate of shell formation using Ca^{45}. *Biol. Bull.* **103**: 269–276.

YOUNG, S. D., M. A. CRENSHAW, AND D. B. KING. 1977a. Mantle protein excretion and calcification in the hardshell clam *Mercenaria mercenaria*. I. Protein excretion in the intact clam. *Mar. Biol.* **41**: 253–257.

YOUNG, S. D., M. A. CRENSHAW, AND D. B. KING. 1977b. Mantle protein excretion and calcification in the hardshell clam *Mercenaria mercenaria*. II. Protein synthesis and excretion by the isolated mantle. *Mar. Biol.* **41**: 259–262.